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# Influence of Organic Buffers on Bacteriocin Production by *Streptococcus thermophilus* ST110

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**Abstract.** The effect of the organic buffer salts MES, MOPS, and PIPES on the growth of *S. thermophilus* ST110, medium pH, and accumulation of the antipediococcal bacteriocin thermophilin 110 were evaluated in whey permeate media over a period of 24 h. In nonbuffered medium, thermophilin 110 production at 37°C paralleled the growth of *S. thermophilus* ST110 and reached a maximum after 8–10 h. Addition of organic buffer salts decreased the drop in medium pH and resulted in increased biomass (dry cells; μg/mL) and higher yields of thermophilin 110 (units/μg cells). The best results were obtained by the addition of 1% (w/v) MES to the medium, which reduced the pH drop to 1.8 units after 10 h of growth (compared to 2.3 pH units in the control) and resulted in a 1.5-fold increase in cell mass (495 μg/mL) and a 7-fold increase in thermophilin 110 yield (77 units/μg dry cells) over the control. The results showed that whey permeate-based media may be suitable for producing large amounts of thermophilin 110 needed for controlling spoilage pediococci in industrial wine and beer fermentations.

Bacteriocins are antibacterial peptides ribosomally synthesized by many types of lactic acid bacteria (LAB; lactococci, lactobacilli, streptococci, and pediococci) used in the industrial production of fermented dairy and meat products, and some bacteriocins exhibit bactericidal activity against Gram-positive bacteria that are commonly associated with food spoilage and food-borne illness. Since the producing microbes are ''generally regarded as safe'' (GRAS), they have attracted particular attention in recent years to develop their potential as natural biopreservatives [6, 23]. Nisin, the lantibiotic bacteriocin of lactococci, is already in use in many countries to control spoilage bacteria and food-borne pathogens such as *Listeria monocytogenes* [4]. The broad-spectrum GRAS category antilisterial bacteriocins pediocin and lacticin 3147 also have the potential for future food applications.

Several strains of the cheese- and yogurt-starter bacterium *Streptococcus thermophilus* produce bacteriocins that display characteristic activity against related strains within this species [1, 14, 17, 26]. However, bacteriocins were also found that are active against listeria [25] and clostridia [18], and the isolation of thermophilin 110, a glycopeptide bacteriocin with a high level of activity against pediococci was recently reported [8]. Because of its unusual antipediococcal activity, thermophilin 110 may be used as a substitute for nisin which was found to be effective in controlling spoilage pediococci in wine and beer fermentations [2, 15, 20, 22].

The production of bacteriocins is usually carried out in complex nutrient media that promote growth and relatively high bacteriocin levels [21]. However, their high cost renders them unsuitable for large-scale bacteriocin production. As an alternative, cheese whey, which is a readily available waste effluent of cheese manufacture, may serve as a growth medium for bacteriocin-producing cultures. The parameters for the

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optimum production in whey-based media (pH, temperature, composition) have been evaluated for several bacteriocins including lacticin 3147 [19], lactocin 705 [24], mesenterocin [5], nisin [7], and pediocin [16], and the production of both nisin and pediocin in whey-based media has also been studied [10, 11].

In this study, we describe the accumulation of biomass and bacteriocin production by *Streptococcus thermophilus* ST110 in whey permeate media in the presence of the nontoxic and minimally penetrating physiological buffer salts MES (2-[*N*-morpholino]-ethane sulfonate), MOPS (3-[*N*-morpholino]-propane sulfonate), and PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonate]) originally introduced by Good et al. [9].

## Materials and Methods

**Bacteria and media.** The *Streptococcus thermophilus* strain ST110 producing thermophilin 110 was grown in tryptone-yeast extract-lactose (TYL) at 37°C [8]. Whey-based media were formulated with whey permeate powder (78–79% lactose content; Davisco Foods International, Inc., Eden Prairie, MN) at 3% (w/v), to yield a lactose concentration of ca. 24 mg/mL, and supplemented with 0.5% (w/v) Difco yeast extract (Becton Dickinson, Sparks, MD). The buffer salts MES, MOPS, and PIPES were purchased from Sigma (St. Louis, MO) and used at 0.5%, 1%, and 2% (w/v) concentrations. All media were adjusted to pH 6.5 and sterilized by filtration through filter cartridge units (Corning) with 0.22-μm cellulose acetate membranes. An overnight culture of *S. thermophilus* ST110 in TYL broth was inoculated into each medium at a concentration of 0.5% (v/v) and growth at 37°C was monitored hourly for up to 12 h by measuring absorbance at 660 nm and converting to dry cell weight from a standard curve. Simultaneously, changes in medium pH were also recorded. The target organism *Pediococcus acidilactici* F (gift from B. Ray, University of Wyoming) used in bioassays was grown at 37°C in deMan, Rogosa, and Sharpe medium (MRS; Becton Dickinson).

**Thermophilin 110 production.** The production of thermophilin 110 by *S. thermophilus* ST110 in whey permeate and control (TYL) media was followed by the spot-on-the-lawn antimicrobial assay of Henderson et al. [12]. Starting at 4 h, culture samples were taken hourly and cells were removed by centrifugation (12,500*g*, 15 min, 4°C). The amount of thermophilin 110 produced was calculated after a twofold serial dilution of cell-free supernatants with sterile distilled $H_2O$, and depositing 5-μL samples on the surface of 2-mm-deep MRS agar plates seeded with 0.5% (v/v) of a 16-h culture of *P. acidilactici.* After storage at 6°C for 6 h the plates were incubated at 30°C for 16 h. The reciprocal of the highest dilution ($2^n$) showing a visible zone of inhibition was defined as the bacteriocin titer. Thus, the thermophilin 110 activity unit (TAU) per milliliter was defined as $2^n \times 1{,}000$ μL/5 μL. The productivity of *S. thermophilus* ST110 in various media is expressed as activity units per microgram of cells (dry weight).

## Results and Discussion

**Growth and thermophilin 110 production by S. thermophilus ST110 in TYL.** Thermophilin 110 is a natural product of the dairy fermentation bacterium *S. thermophilus* ST110 [8] with a uniquely high antimicrobial activity against pediococci (Fig. 1) that are recognized as important spoilage organisms in wine and beer fermentations [2, 15]. The potential for industrial applications implies a need for improving the yield of this food-grade bacteriocin under defined conditions using relatively inexpensive media such as whey permeate.

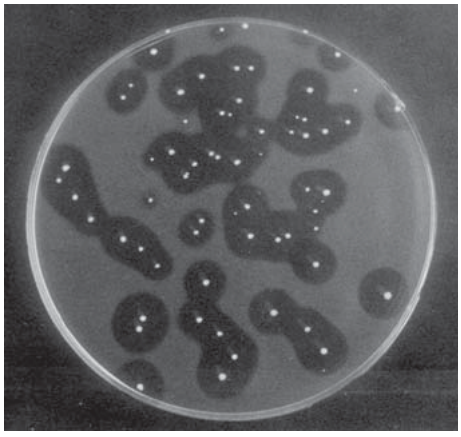

Fig. 1. Bacteriocin production by *S. thermophilus* ST110. Zones of inhibition developed in an MRS agar plate with *P. acidilactici* as the target organism when overlaid with *S. thermophilus* ST110 colonies grown on TYL agarose/agar films for 36 h.

The growth and productivity of *S. thermophilus* ST110 were evaluated in both conventional (TYL) and whey permeate-based media. Since previous studies already demonstrated the importance of controlling medium pH in the whey-based production of nisin [7] and pediocin [11, 16], the effects of the physiological buffer salts MES ($pK_a$ 6.15), MOPS ($pK_a$ 7.2), and PIPES ($pK_a$ 6.8) were also tested on biomass accumulation and thermophilin 110 production by ST110. In contrast to phosphate, which is commonly used as a medium ingredient and serves not only as a buffering agent but also as an active participant in many biochemical reactions, the Good buffer salts do not readily pass through cell membranes and will not accumulate within cells to interfere with physiological processes [9]. Preliminary experiments showed that MES, MOPS, and PIPES did not inhibit the growth of ST110 when added to conventional media (TYL) up to a 2% (w/v) final concentration.

*S. thermophilus* ST110 is a typical LAB that may be maintained in several enriched microbiological media. During growth in the nutrient-rich but nonsupplemented TYL control medium (initial pH 6.5) at 37°C, *S. thermophilus* ST110 cultures as a rule reached a maximum OD of 1.2–1.3 (measured at 660 nm) within 8 to 10 h, which on the average corresponded to a biomass of 400–425 μg/mL (dry weight). As with other LAB, the

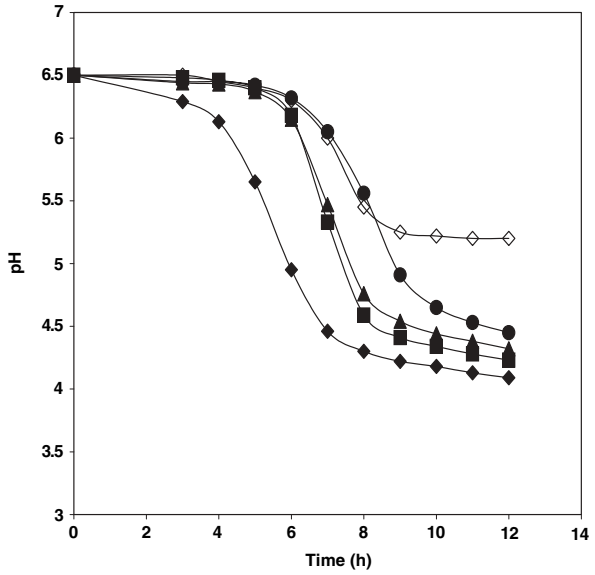


Fig. 2. Time course of pH during the growth of *Streptococcus thermophilus* ST110 in nonbuffered (♦) and buffered (●, 1% MES; ■, 1% MOPS; ▲, 1% PIPES) whey permeate media. Control medium: TYL (◇).

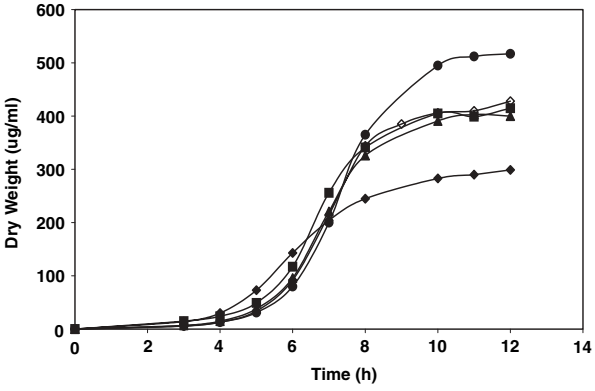


Fig. 3. Time course of biomass accumulation (μg/mL) during the growth of *Streptococcus thermophilus* ST110 in nonbuffered (♦) and buffered (●, 1% MES; ■, 1% MOPS; ▲, 1% PIPES) whey permeate media. Control medium: TYL (◇).

accumulation of lactic acid produced lowered the medium pH to 4.8–5.2, which creates an unfavorable environment for further growth. Therefore, extension of incubation period to 24 h resulted only in incremental gains in biomass (<10%). During the 8- to 10-h incubation period the thermophilin 110 titer reached a maximum of 3,000–4,000 TAU/mL, followed by a gradual decline in detectable activity. At the time of the highest detectable level of thermophilin 110, the productivity of the culture was calculated as ca. 8–9 TAU/μg dry cell weight (see Figs. 2–4 and also Table 1).

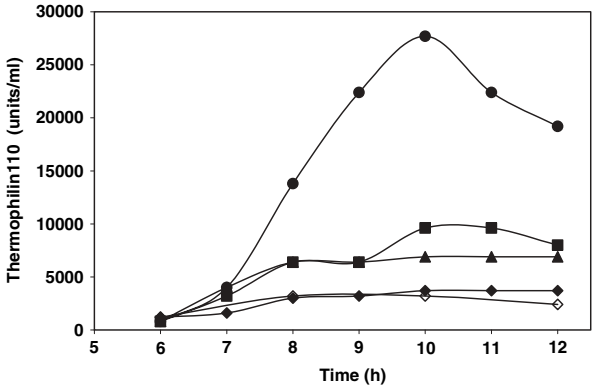


Fig. 4. Production of thermophilin 110 during the growth of *S. thermophilus* ST110 in nonbuffered (♦) and buffered (●, 1% MES; ■, 1% MOPS; ▲, 1% PIPES) whey permeate media. Control medium: TYL (◇).

Table 1. Thermophilin 110 production: TAU units per microgram of cells (dry weight)

| Time (h) | TYL | WP | WP+ 1% MES | WP + 1% MOPS | WP + 1% PIPES |
|---|---|---|---|---|---|
| 8 | 9.2 | 13.0 | 26.3 | 8.7 | 19.5 |
| 10 | 7.8 | 11.3 | 77.0 | 31.6 | 16.6 |
| 12 | 3.8 | 10.7 | 50.0 | 30.8 | 16.0 |
| 24 | 2.0 | 6.6 | 28.5 | 13.8 | 8.5 |

*Note*. TYL, tryptone-yeast extract-lactose; WP, whey permeate.

The addition of the physiological buffer salts MES, MOPS, or PIPES to the TYL medium resulted in slightly higher pH values over the control (pH 5.2) after 10 h of growth of ST110. At 0.5%, 1%, and 2% (w/v) final concentrations, the corresponding pH values were 5.65, 5.95, and 6.15 for MES, 5.65, 5.9, and 6.15 for MOPS, and 5.35, 5.58, and 5.95 for PIPES. Further, the addition of Good's buffer salts to TYL did not have a significant impact on either the biomass generated or the amount of thermophilin 110 produced (3,200 TAU/mL).

**Growth and thermophilin 110 production in whey permeate.** Media prepared from filter sterilized whey permeate (WP) without added yeast extract apparently lacked the growth factors needed by ST110 to attain high cell densities. After 10 h of incubation, the medium pH dropped to 4.15 but the biomass accumulated was only 210 μg/ml (dry weight), and antimicrobial assays with *P. acidilactici* showed only a trace level of bacteriocin in cell-free supernatants. These results were similar to that reported previously on the requirement for supplementing WP with yeast extract to attain optimum production of pediocin [16].

The supplementation of WP media with 0.5% yeast extract (WPY) resulted in substantial increases in both biomass and the amount of bacteriocin produced by ST110. In three replicate trials, starting at 3 h after inoculation, samples were taken at 60-min intervals to check $OD_{660}$, pH, biomass accumulation (dry weight), and antimicrobial activity (TAU/ml). As in TYL, biomass production in WPY also peaked after approximately 10 h of incubation (300 μg/mL) and thermophilin 110 was also at the highest level (3,700 TAU/mL) when the medium pH dropped to 4.2. Although the amount of biomass produced after 10 h at 37°C was ca. 25% lower than that found in TYL, the productivity of ST110 in WPY was 11.3 TAU/ μg biomass (dry weight), corresponding to a >40% increase over that found in TYL. These results are shown in Figs. 2–4 and also in Table 1.

**Effect of buffer salts on growth and thermophilin 110 production in whey permeate.** The addition of MES, MOPS, or PIPES to WPY medium at a 0.5% (w/v) concentration had a negligible effect on medium pH over the course of a 12-h growth period compared with the WPY control. However, at concentrations of 1% or higher, the buffering capacity of all three buffer salts became noticeable. As shown in Fig. 2, MES ($pK_a$, 6.15), with the highest buffering capacity between pH 5.5 and pH 6.7, was more efficient in delaying the decline in medium pH than MOPS and PIPES, which have an optimum pH range of 6.5–7.9 and 6.1–7.5, respectively.

The presence of MES, MOPS, or PIPES in WPY medium at a 1% or 2% concentration had a significant impact on the growth of ST110 and contributed to increases in biomass accumulation. After 10 h of incubation, the dry weight of biomass in WPY-MES, WPY-MOPS, and WPY-PIPES (each buffer salt at 1%, w/v), was 495, 405, and 390 μg/mL, respectively, while the ST110 biomass in nonbuffered WPY was 300 μg/ml (Fig. 3).

The addition of Good's buffer salts also influenced the amount of bacteriocin produced by ST110. Since all three buffer salts increased the yield in biomass, a corresponding increase in thermophilin 110 titers (TAU/mL) was expected. After 10 h of incubation, the amount of thermophilin 110 produced was 9,600, 8,000, and 27,000 TAU/mL for media supplemented with 1% MES, MOPS, and PIPES, respectively (Fig. 3), representing 7-fold, 2.5-fold, and 2-fold increases over the thermophilin 110 titer of the control WPY medium (3,700 TAU/ml). However, the greatest impact of the buffer salts was related to the productivity of *S. thermophilus* ST110, i.e., the amount of TAU produced per microgram dry cell weight. The calculated productivity values for the ST110 culture grown in WPY-MES, WPY-MOPS, and WHY-PIPES were 7-fold, 3-fold, and 1.5-fold higher than the productivity of ST110 in WPY control, and even higher compared with ST110 grown in conventional TYL medium, as shown in Table 1.

It has been repeatedly demonstrated that the pH of the growth medium has a strong influence on bacteriocin production by LAB. Thus, an optimum pH for nisin production by *Lactococcus lactis* was reported to be in the pH 5.5–6.1 range [13], pediocin was produced optimally by *Pediococcus acidilactici* at a medium pH below 4.5 [3], and lactocin 705, a bacteriocin of *Lactobacillus* casei CRL 705, was best produced in the pH range 6.5–7.5 [24]. Although in this study provisions were not included for maintaining pH at a preselected level over the entire course of each trial, the buffer salts MES, MOPS, and PIPES were effective in reducing the rate of decrease in medium pH. Apparently, a medium pH higher than 4.5 was more favorable for bacteriocin production by *S. thermophilus* ST110.

In conclusion, WP supplemented with a moderate amount of yeast extract (0.5%) supported growth and bacteriocin production by *S. thermophilus* ST110. Although the yields of thermophilin 110 were comparable (3,700 TAU/mL), the biomass accumulated after 10 h growth in WPY was only 70% of that found in the more complex TYL medium (400 μg/mL), and corresponded to a 43% increase in culture productivity (TAU/μg of dry weight).

The addition of the nontoxic physiological buffer salts MES, PIPES, and MOPS (Good's buffers), which are characterized by minimal penetration of cell membranes, further improved the bacteriocin yields of ST110. The highest level of thermophilin 110 production (27,000 TAU/mL) was achieved by the addition of 1% MES, which represented a nearly 7-fold and 10-fold increase in culture productivity over the control values determined for ST110 grown in WPY and TYL media.

The results showed that WP may serve as an excellent medium for producing large amounts of the antipediococcal bacteriocin thermophilin 110 with potential for applications in controlling spoilage-causing pediococci in industrial fermentations.

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